*Soil Biol. Biochem.* Vol. 22, No. 2, pp. 187–194, 1990
Printed in Great Britain

0038-0717/90 $3.00 + 0.00
Pergamon Press plc

# COMPARISON OF BIOMASS AND VIABLE PROPAGULE MEASUREMENTS IN THE ANTAGONISM OF *TRICHODERMA HARZIANUM* AGAINST *PYTHIUM ULTIMUM*

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(*Accepted* 25 *August* 1989)

**Summary**—A potting medium was used to demonstrate the biological control potential of *Trichoderma harzianum* against damping-off of lettuce (*Lactuca sativa*) caused by *Pythium ultimum*. Introduction of *T. harzianum* but not *P. ultimum* into the medium caused an immediate increase in the ATP and chitin contents followed by a rapid decline. By contrast, initial increases in ergosterol concentration and esterase activity on fluorescein diacetate were followed by inconsistent and extremely variable responses with duration of incubation. The colony-forming units (cfu) of *Trichoderma* increased on amendment and were sustained whereas the increased units of the *P. ultimum*-only treatment were reduced by *T. harzianum*. Respiration increased for 2 days after amendment and then declined. It is concluded that ATP and chitin measurements are suitable biomass markers for introduced fungal inocula and are inversely associated with cfus and esterase-activity measurements. Both types of measurement are useful in investigating disease potential and biocontrol activity. Biocontrol activity is primarily linked to a transient increase in *Trichoderma* biomass resulting in a sustained increase in active propagules of the antagonist.

## INTRODUCTION

Microbial biomass in soil is composed primarily of bacterial cells and fungal mycelia and spores. The total biomass of both groups of microorganisms, especially the biomass important in ecological interactions, is difficult to assess. Several methods have been described for estimating fungal biomass, but each has its own limitations and difficulties (Söderstrom, 1979; Schnurer and Rosswall, 1982; Whipps *et al.*, 1982; Matcham *et al.*, 1984; Martens, 1987; Baath, 1988; Chen *et al.*, 1988). Among the limitations are:

(1) difficulty in distinguishing between fungal and bacterial biomass;
(2) separating living, senescent and dead cells; and
(3) measuring biomass in solid and opaque substrates, such as soil.

In microecological studies in soil it is necessary to determine the relative activity and importance of components of microbial biomass, e.g. there is considerable interest in manipulating the soil microbial community to achieve the biological control of soilborne plant pathogens. Antagonistic fungi and bacteria have both been used (Cook and Baker, 1983), and *Trichoderma* spp have been of special interest (Papavizas, 1985).

Our objective was to assess the relative effectiveness of several methods of estimating fungal biomass in a biological control system in which *Trichoderma harzianum* Rifai was used to control damping-off of lettuce (*Lactuca sativa* L.) caused by *Pythium ultimum* Trow in a glasshouse production system. These methods were compared to measurement of viable propagules (colony-forming units: cfu) which has been widely used as an indication of fungal survival and colonization.

## MATERIALS AND METHODS

### *Biocontrol assay system*

A potting mix ("mix") utilized commercially for glasshouse production of lettuce was used to assess biological control of *P. ultimum*-induced damping-off of lettuce (*L. sativa* L., cv. Winter Bunch). The isolate of *P. ultimum* used was PuMXL, obtained from a lettuce plant with root rot. The non-sterile mix (pH 6.8) consisted of 50:50 sand:peatmoss (v/v) amended with chalk (0.62 g $l^{-1}$), macronutrients ($KNO_3$ 0.38 g $l^{-1}$ and superphosphate 0.77 g $l^{-1}$) and micronutrients (Frit WM 255, FENO Chemical Division, 400 mg $l^{-1}$) according to accepted horticultural practice. The mix was infested with 200 sporangia $g^{-1}$ mix of *P. ultimum* and amended with the *T. harzianum* [isolate IMI 275950, from wheat straw

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(Lynch, 1987)] inoculum described below, at a concentration of 1% (w w) immediately before planting. The pots (7.5 cm dia) were planted with 10 lettuce seeds in four replicate pots for each treatment. The pots were placed on capillary matting in a glasshouse which was maintained between 15–25 C and watered routinely as required. After 14 days, measurements of plant emergence (final stand), plant dry weight and maximum root length were made.

*Production of inoculum*

Sporangia of *P. ultimum* were produced by modification of the procedure of Ayers and Lumsden (1985) in which 3 day old cultures on Difco cornmeal agar (CMA) were flooded with sterile 10% soil extract. After growth for 1–2 weeks, mycelia containing sporangia were scraped from the agar surface, and blended in a laboratory mixer (Silverson laboratory mixer-emulsifier, Waterside, Chesham, Bucks., England) at full speed for 1 min. Sporangia were counted in a haemacytometer and diluted to deliver 200 sporangia $g^{-1}$ dry weight equivalent of mix.

Inoculum of *T. harzianum* was prepared (Papavizas *et al.*, 1984) by growing the antagonist in shaken flask (capacity 1 l.) culture at 150 rev $min^{-1}$ in a medium containing 15 g molasses and 2.5 g dried yeast in 500 ml distilled water for 5 days at room temperature (25 C). Mycelia and spores were harvested by filtration under vacuum through coarse muslin cloth. The mycelial mats were air dried for 1 week and ground ($< 500\ \mu m$) in a Cyclotec sample mill (Tecator, Bristol) laboratory grinder and stored at 4 C. Preparations were assayed for cfus before use. Free spores and particles containing chlamydospores were also counted with a haemacytometer. The cfu contained in 1 g of dried biomass was $4 \times 10^7$ viable propagules composed primarily of chlamydospores, compared to $3 \times 10^6$ actual countable spores or spore aggregates.

*cfu Measurements*

cfu Measurements for *T. harzianum*, assayed by the most-probable-number (MPN) technique (Harris and Sommers, 1968) were determined from serial dilutions of 1.0 g samples of mix in 9 ml sterile distilled water containing 0.2% agar. Eight drops of suspension (50 $\mu$l) in 0.2% water agar were applied to one-half of a Petri plate of TME medium (Papavizas and Lumsden, 1982) for each dilution. The plates were kept under light for 5 days at 25 C and positive droplets counted and coverted to cfu from a standard MPN table (Harris and Sommers, 1968).

cfu Of *P. ultimum* were estimated by the same method as above but 4-fold dilutions were applied onto the surface of a *Pythium* selective medium (Tsao and Ocana, 1969). This medium (VP medium) contained Vancomycin (200 mg $l^{-1}$), Pimaricin (10 mg $l^{-1}$) and pentachloronitrobenzene (100 mg $l^{-1}$) in Difco cornmeal agar. The VP plates were kept for 24 h at 20 C. Colonies developing from the droplets within 24 h were identified as *P. ultimum*.

*Biomass determinations*

All the techniques were used in individual biocontrol experiments at least twice. A single composite experiment, using all the techniques for biomass measurement, is described. Three treatments were used:

(a) *P. ultimum* incorporated into the mix at 200 sporangia $g^{-1}$;

(b) *P. ultimum* at the same concentration as in (a) plus *T. harzianum* incorporated at a concentration of 1% on an equivalent dry weight basis and;

(c) a healthy control without additions of inoculum or amendment. All pots were sown with lettuce and maintained as described for the biocontrol assay system. Pots were destructively sampled at 0, 2, 4, 8 or 14 days after planting.

*Adenosine triphosphate (ATP) estimations.* Samples of mix (1 g) were removed at the appropriate time and frozen immediately to −20 C. Subsequently, the frozen mix was homogenized with 50 ml of Tris–EDTA buffer (37.7 g Tris, 1.5 g EDTA in 1 l. polished water) and ATP estimated (Eiland, 1985). Water was polished by reverse osmosis, organic absorption and deionization in an Elgastat Spectrum Water Purifier (Elag Ltd, High Wycombe, Bucks., England). All reagents used were obtained from SONCO (Upper Bately, Yorks., England) except for the ATP standard (Sigma, Poole, Dorset, England). The resulting suspension (100 $\mu$l) was pipetted into a luminometer cuvette, 100 $\mu$l of the nucleotide releasing agent (NRB) were added, the cuvette was incubated for 20 s, then 100 $\mu$l of LUMIT PM were added and the reading from the luminometer taken after a 10 s integration of the signal. An internal standard of 20 $\mu$l of an ATP standard was immediately injected and a second reading taken.

*Fluorescein diacetate (FDA) esterase activity.* FDA-esterase activity was determined using a system based on the method of Swisher and Carroll (1980). Samples of mix for FDA-esterase measurements were obtained from the first and second 10-fold dilutions in 0.2% water agar described above for CFU counts. A 1 ml sample of each dilution was added to 4.0 ml potassium phosphate buffer in centrifuge tubes, providing a final concentration of 50 mM buffer, pH 7.0. To the above mixture was added 2 $\mu$l FDA stock solution containing 2.0 g $l^{-1}$ FDA in acetone. The stock solution was stored at −10 C until used. After addition of the FDA, the tubes were shaken for 2 h at room temperature on a platform shaker at 300 rev $min^{-1}$. The tubes were placed in an ice bath and subsequently centrifuged at 3000 rev $min^{-1}$ for 10 min at 0 C. The optical density (OD) of the supernatant solution was measured at 490 nm in a Pye Unicam SP 30 u.v. Spectrophotometer (Pye Instruments, Cambridge, Cambs., England) and the esterase activity expressed as OD units $g^{-1}$ dry mix.

*N-acetyl glucosamine (chitin) estimations.* The techniques used were modified from those described by Whipps (1987) based on the methods of Ride and Drysdale (1972). Samples of mix (10 g) were oven-dried at 80 C and ground ($< 500\ \mu m$) in a Cyclotech 1030 sample mill. Four replicate samples (100 mg dry wt) of mix for each treatment at each harvest were placed in Pyrex sovirel tubes and were washed once with acetone (5 ml) by shaking for 20 min and centrifuging (6000 *g*, 10 min, 2 C). The acetone was decanted and the pellet washed twice with distilled water. After the final centrifugation, the supernatant

was removed and 120% w/v KOH (3 ml) was added. The tubes were sealed and placed in an oven at 130°C for 1 h. After cooling, the tubes were placed on ice. Celite suspension (0.9 ml), prepared by mixing 1 g Celite 545 with 20 ml ice-cold 75% ethanol and standing for 2 min, was layered onto the surface and the tubes were centrifuged (6000 *g*, 10 min, 2°C). The supernatant was discarded and the pellet containing the chitosan preparation was washed sequentially with ice-cold 40% ethanol, and distilled water (twice). The pellet remaining after the washing treatment was then assayed for "chitin" content by a colorimetric system using glucosamine as standard (Ride and Drysdale 1972).

*Ergosterol estimations.* The ergosterol extraction and quantification was a modification of the method used by Seitz *et al.* (1979). Samples of mix (30 g fresh wt) were sonicated for 1 min at maximum power on a Rapid ultrasonic disintegrator (Ultrasonics Ltd, Bradford, W. Yorks., England) in 40 ml of the extraction solvent of methanol:dichloromethane (2:1, v/v). After 30 min the suspension obtained was filtered under vacuum (Whatman No. 3 filter paper) and the residue washed with 40 ml dichloromethane followed by 40 ml of 2 M KCl in 0.5 M phosphate buffer (pH 7.4). The filtrate and the washings were combined and the aqueous phase was discarded. The organic phase was evaporated to dryness under reduced pressure at 40 C in a rotary evaporator. The solid obtained was stored overnight at 4 C in sealed flasks. The solid was saponified in 30 ml 1 M KOH in 95% ethanol (v/v) for 1 h at 70 C. Once cool, 60 ml of distilled water was added and the non-saponified fraction was extracted with three washings of petroleum ether. The three washings were combined and evaporated to dryness under reduced pressure at room temperature in a rotary evaporator. The product obtained was stored under nitrogen in sealed tubes at 4°C until required. The ergosterol in the extract was quantified by reverse phase HPLC (Chrompack C18 cartridge fitted to a Walters Model 6000A solvent delivery system and Model U6K liquid chromatograph injector). The solid extract was dissolved in 2 ml of methanol at 60°C and centrifuged in a bench top centrifuge for 10 min before loading onto the column. A solvent system of methanol:water (95:5, v/v) provided good separation of the ergosterol peak and a relatively short assay time. The absorbance of the eluant from the column was monitored at 280 nm by a Pharmacia u.v.-1 single path u.v. monitor. The ergosterol peak was identified from the trace by adding an ergosterol standard (Sigma, Poole, Dorset, England) to the samples. A calibration curve was produced using the same ergosterol standard.

*Respiration ($CO_2$) estimations*

To avoid complications with plant respiration the pots for respiration measurements were not sown with lettuce. On four sampling days replicate pots of each treatment were placed individually into Kilner jars of known volume (*ca* 500 ml) and sealed with an airtight lid. Every hour for up to 5 h a sample (1 ml) of headspace gas was withdrawn through a septum in the lid of the jar. The $CO_2$ concentration was determined using an Alltech CTR1 column and a thermal conductivity detector in a Shimadzu GC-8A gas chromatograph (Burton *et al.*, 1987). The chromatograph was calibrated using standard gas mixtures from Phase Separation (Queensferry, Clywd). After 14 days the pots were analysed for bulk volume and dry weight of mix and respiration rates ($\mu$g $CO_2$ evolved $h^{-1}$ $g^{-1}$ dry wt of mix) were calculated.

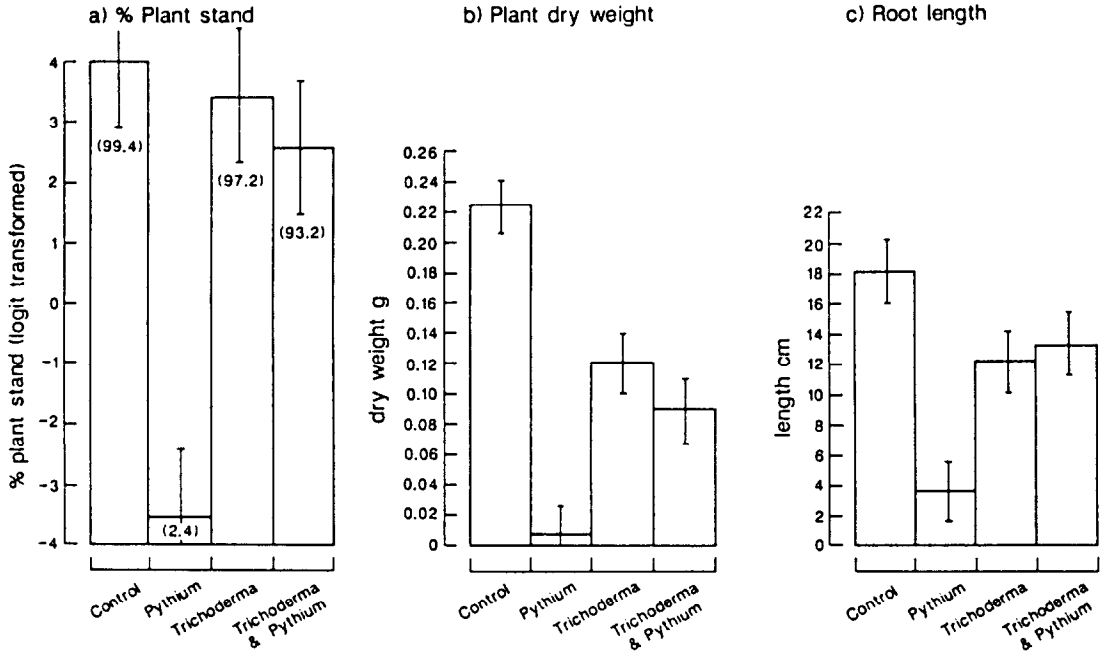


Fig. 1. Biocontrol of *P. ultimum* damping-off of lettuce by *T. harzianum* after 14 days: (a) % plant stand, (b) plant dry weight and (c) maximum root length. Error bars are SED $[\log(x + 0.5/100.5 - x)] \pm$ SED.

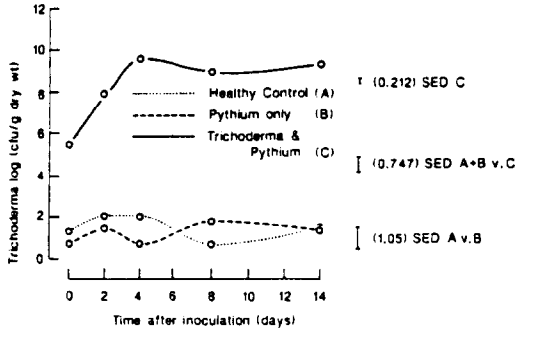


Fig. 2. Changes in *Trichoderma* population in the mix.

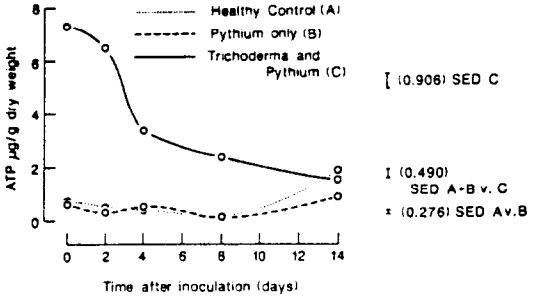


Fig. 4. Changes in ATP content of the mix.

*Statistical analysis*

For the cfu results ($x$), analyses of variance was performed on a log transformation of the data as log $(x + 1)$.

The plant measurements expressed as percentage plant stand ($y$) were transformed using a logit transformation $[Y = \log(y + 0.5/100.5 - y)]$ and the transformed data were analyzed using analysis of variance. Analysis of variance was used for other data.

## RESULTS

*Plant growth and biological control with T. harzianum*

*T. harzianum* effectively reduced the incidence of damping-off caused by *P. ultimum* in commercial glasshouse potting mix, producing degrees of disease control that were not significantly different from the percentage plant stand of the uninoculated control (Fig. 1). *T. harzianum* alone did not affect plant stand.

The addition of *T. harzianum* to *P. ultimum* significantly reduced the dry weight loss and reduction in root length caused by *P. ultimum* alone, but these improvements were still significantly lower than the non-inoculated healthy control. *T. harzianum* alone reduced plant dry weight and root length.

*Fungal populations*

Analysis of cfus of *Trichoderma* showed that there were significant differences between the main effects of the three treatments with the number of cfus in the *Trichoderma* + *Pythium* treatment being significantly greater than those found in the control and *Pythium*-only treatments (Fig. 2). Analysis of the *Trichoderma* + *Pythium* treatment alone, showed that there were significant differences between the number of cfus recorded with time. The cfus increased from an initial amount at amendment of *ca* $1 \times 10^5$ cfu $g^{-1}$ to *ca* $1 \times 10^9$ cfu $g^{-1}$ after 4 days and remained at that level for at least 10 days. Associated with the increase in cfus of *Trichoderma* was a proliferation of white mycelium on the surface of the mix, which later became green as conidiation occurred.

The cfus of *Pythium* showed that there were significant differences between the main effects of the three treatments, where the *Pythium*-only treatment had significantly more *Pythium* cfu present throughout the whole experiment (Fig. 3). Analysis of the *Pythium*-only treatment showed that there were significant differences present in the number of cfus recorded with time. There were fewer cfus at day 2, but the numbers after day 4 remained constant.

The number of detectable *Pythium* cfus in the *Pythium* + *Trichoderma* treatment was significantly reduced by day 2, and remained below the *Pythium* alone treatment at 14 days (Fig. 3). The total biomass contributed by *P. ultimum* was comparatively very small.

*Biomass determinations*

*ATP.* Analysis of extractable ATP concentrations showed that there were significant differences between the main effects of the three treatments, where the *Trichoderma* + *Pythium* treatment showed a decline in the amounts of ATP (from 6.6 µg $g^{-1}$ to 1.1 µg $g^{-1}$) over 14 days (Fig. 4). Analysis of the *Trichoderma* + *Pythium* treatment showed that the amounts on day zero and day 2 were significantly greater than those recorded after day 4. ATP concentrations in the non-amended control and *Pythium*-only treatments were not significantly different from

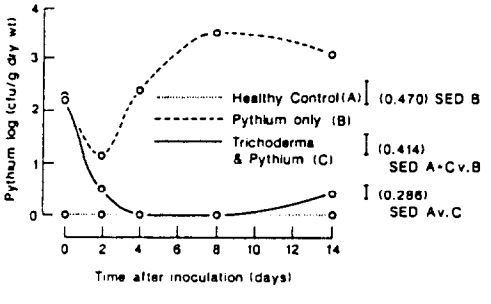


Fig. 3. Changes in *Pythium* population in the mix.

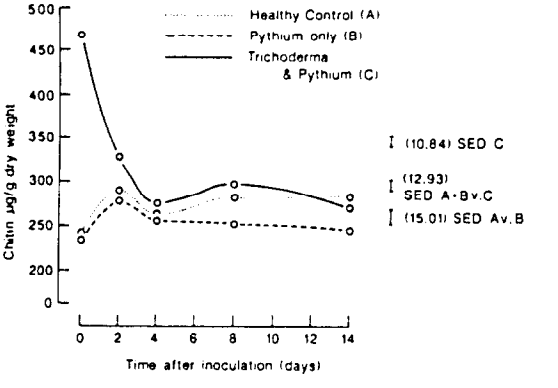


Fig. 5. Changes in chitin content of the mix.

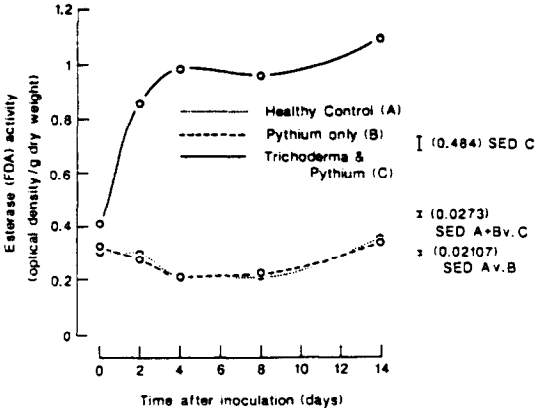


Fig. 6. Changes in esterase activity of the mix.

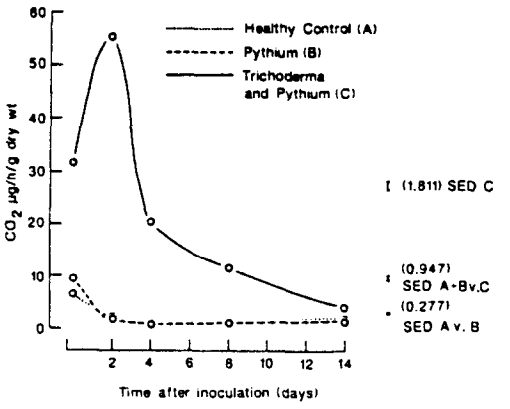


Fig. 8. Changes in respiration of the mix.

each other and remained relatively constant throughout the experiment, despite the high levels of variation observed for some of the values recorded.

*Chitin.* Analysis of the three treatments showed that there were significant differences between the chitin levels recorded and that the amounts of chitin in the *Trichoderma* + *Pythium* treatment showed a significant decline, from day zero to similar amounts as the other treatments by day 4 (Fig. 5). Chitin concentrations in the healthy control and *Pythium* only treatments showed significant differences between the main effects of treatment and time, but both treatments showed similar chitin amounts throughout the 14 day period.

*FDA esterase activity. T. harzianum* biomass, expressed as OD $g^{-1}$ dry mix read at 490 nm, showed variable results in two separate experiments. In the first, *T. harzianum* amendment was associated with a rapid decrease in OD after day 2 (data not shown). In contrast, a repeat experiment (Fig. 6) showed an opposing response. In this case, the readings increased from *ca* OD 0.4 $g^{-1}$ dry mix to *ca* 1.0 by day 4 with constant readings thereafter to day 14.

*Ergosterol.* Detection of ergosterol was extremely variable during the period of assay of the mix in the *Pythium* only and *Trichoderma* + *Pythium* treatments. Subsamples were variable from replicate sample to sample and standard deviations were large. The ergosterol amounts recorded for the control and *Pythium* treatments remained low and showed no significant differences (Fig. 7). Values for the *Trichoderma* + *Pythium* treatments increased from 10 µg $g^{-1}$ dry wt at zero time to 110 µg $g^{-1}$ dry wt at day 4, then decreased thereafter to 15 µg $g^{-1}$ dry wt at day 14. To further check the data recorded for the *Trichoderma* + *Pythium* treatment, the data were analysed using a *t*-test at each time point to see if the mean values were significantly greater than zero. The means calculated at each of the 5 time points were found to be significantly greater than zero at the 5% level.

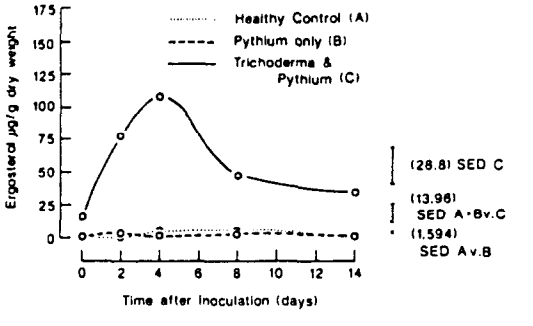


Fig. 7. Changes in ergosterol content of the mix.

*Respiration as determined by $CO_2$ release.* Analysis of the amounts of $CO_2$ (Fig. 8) showed that there were significant differences between the effects of the 3 treatments. This was largely due to a burst of $CO_2$ evolution from zero time with the *Pythium* + *Trichoderma* treatment, which showed that there were significant differences between each of the time points with a burst of $CO_2$ from zero time to day 2, followed by a sharp decline in $CO_2$ from day 4 to 14. Analysis of the control and *Pythium* treatments showed that there were differences between the 2 treatments at day zero where the $CO_2$ evolution was slightly elevated, possibly because of physical disturbance of the mix, the values then levelled off to a fairly constant low rate of respiration thereafter.

## DISCUSSION

*T. harzianum* effectively reduced the incidence of damping-off of lettuce by *P. ultimum* in the mix in these pot tests. However, plant dry weight and root length were still less than the healthy control in the presence of *T. harzianum* whether *Phythium* was present or not. Evidently the *T. harzianum* strain used at 1% w/w basis reduced plant growth somewhat. This may be related to the production of volatile pentyl and pentenyl-pyrones by this fungus (Claydon *et al.*, 1987) which, besides being fungistatic (and effecting biocontrol action) can have phytotoxic side-effects at high doses.

That small values were obtained for all the assays of microbial biomass for healthy controls and pathogen only treatments implies that the bulk of the biomass detected in the *Trichoderma* + *Pythium* treatments was derived from *T. harzianum* (Figs 4–8). However, other than chitin which is absent from Oomycetes, none of the biomass markers used can be regarded as specific for the pathogen or antagonist introduced. Bacteria and microfauna are positive in

the chitin, ATP, esterase and respiration assays. But biomass has been measured comparatively in these experiments and the primary interest was the changes in response to the addition of the relatively massive 1% w w *Trichoderma* preparation. Nevertheless, there was little general consistency in the pattern of changes in various measurements of microbial biomass in the *Trichoderma* treatments with time. ATP and chitin values decreased steadily, FDA activity increased and remained high whereas ergosterol and $CO_2$ production peaked and then declined.

The chitin values in the *Trichoderma* treatment fell with time even though the mix turned green as the *Trichoderma* sporulated. The chitin assay used in these experiments has been utilized successfully to monitor growth of the ectomycorrhizal fungi *Laccaria laccata* and *Suillus bovinus* in peat-vermiculite (Whipps, 1987) and may be considered fairly reliable in such artificial peat-based mixes, even though chitin assays using other extraction and estimation techniques are thought to be less suitable than other biomass estimation techniques in soil systems (West *et al.*, 1987; Grant and West, 1987).

The results of the FDA esterase activity and ergosterol assays, shown in Figs 6 and 7 respectively, did not follow the pattern of the chitin and ATP assays. The FDA esterase activity assay relies on the uptake of the substrate and release of the product being the same for mycelium of all ages and the amount of esterase present at all growth stages being constant. During this experiment, *Trichoderma* grew vegetatively and sporulated and then probably autolysed. It seems possible that the amount of esterase present could vary at these different stages of growth and development. The FDA-esterase activity showed no correlation with the respiration results (Fig. 8). However, this is perhaps not surprising as respiration reflects the activity of a much wider group of enzymes than the esterases. The variability of the assay limits its potential as a biomass marker in this system but FDA-esterase activity has been correlated with total microbial biomass based on extractable phospholipid-phosphate (Chen *et al.*, 1988). FDA-esterase activity has also been useful for indicating *Trichoderma* biomass in pure culture (Veal and Lynch 1987). Thus FDA-esterase activity may be a reliable assay under certain well-defined conditions only.

Although ergosterol is a highly-specific chemical marker for fungi, its use as a biomass marker for *T. harzianum* in this study was limited because of the great variability between replicate samples although there was little variation betwen replicate extractions of the same sample. This contrasts with the findings of West *et al.* (1987) which showed ergosterol to be an accurate soil fungal biomass marker. There is, however, a big difference between the two systems studied. West *et al.* (1987) examined undisturbed soils containing a mixed population of fungi but in our study the total fungi biomass was dominated by one fungus which had recently been introduced into the soil mix. Any fluctuations of ergosterol content of the mycelium with age or growth stage would be much more obvious in our system which was followed over a period of days. It is well documented that ergosterol content in mycelium will vary at different growth stages (Dehorter *et al.*, 1980; Evans and Gealt, 1985). There seems to be no general pattern for the developmental regulation of this sterol. Major changes in ergosterol content have been associated with the induction of sporulation (Dehorter *et al.*, 1980), the ageing of mycelium (Nout *et al.*, 1987), the growth medium (Miller *et al.*, 1985) and autolysis of mycelium (Newell *et al.*, 1987). The ergosterol content peaked at day 4 in this experiment (Fig. 7). The increase in ergosterol when the biomass was thought to be declining (based on ATP and chitin measurements) took place when the mycelium could be seen to be sporulating, and the *Trichoderma* cfus were increasing (Fig. 2). A change of ergosterol content with age and sporulation would explain why the ergosterol assay was not directly related to ATP and chitin measurements in this system.

Respiration of *T. harzianum*-treated pots followed a pattern similar to that observed after addition of conidia of *T. viride* to a steamed potting mix based on peat, perlite and sandy loam (Marois and Locke, 1985). In that study, nutrients were released from the soil by the steaming treatment. Respiration also increased in soils amended with mycelial preparations of *Trichoderma* spp on bran (Lewis and Papavizas, 1984a). Increased microbial activity as measured by high respiratory activity has repeatedly been correlated with disease suppression (Alabouvette *et al.*, 1985). Perhaps in these systems the *Trichoderma* was able to outcompete the *Pythium* for substrate priority either for germination or infection.

It is difficult to reconcile such variable responses in biomass estimation to the visual observation of fungal growth and sporulation seen on the pot surface. Nevertheless, after consideration of each of the assays of microbial biomass, it may be possible to produce a coherent hypothesis. When the *Trichoderma* preparation is incorporated into the pot it represents a finite amount of resource which supplies most of the energy for subsequent germination and growth of the accompanying chlamydospores of *Trichoderma* (Lewis and Papavizas, 1984b) as well as the indigenous microflora of the mix in contact with it. The *Trichoderma* preparation consists mostly of chlamydospores, the remainder being dead fungal mycelium rich in chitin and other cell wall compounds, much of the soluble material having been utilized during the drying stage before incorporation. Thus, *ca* 90% of the *Trichoderma* preparation is available as substrate for growth of the *Trichoderma* and the indigenous microflora. However, from an ecological point of view, the *Trichoderma* has an advantage in comparison with the indigenous microflora as it is immersed within the substrate. Consequently, immediately on incorporation the chlamydospores perhaps germinate, utilize any immediately assimilable materials such as sugars and amino acids and then rapidly switch over to degradation of cell wall material for growth. *Trichoderma* spp are known to utilize fungal cell walls as a carbon source and the presence of fungal cell walls rapidly induces the production of chitinase and other cell wall degrading enzymes in this fungus (C. J. Ridout, unpublished Ph.D. thesis; Ridout *et al.*, 1986). This would provide energy for new mycelial growth. Consequently $CO_2$ production would be

high, FDA-esterase activity would increase but total chitin values in the mix would decrease.

Similarly, the total ATP of the viable fungal system may be decreasing if there was a greater quantity of ATP stored within the chlamydospores compared with the new vegetative mycelium. Subsequently, substrate limitation would occur and a switch to sporulation would take place. Respiration would decrease, FDA esterase activity would stabilize if spore numbers did not change and the amounts of chitin and ATP would reach the concentrations in the background. Ergosterol contents could increase at the time of sporulation when both mycelium and spores contained ergosterol but would decline when only the spores remained viable and the vegetative mycelium autolysed.

The biomass information provided by these experiments poses interesting questions as to how the biocontrol was achieved. It is possible that the events leading to biocontrol of *Pythium* could have taken place very soon after the introduction of the *Trichoderma*. Consequently, high numbers of *Trichoderma* need not be present to achieve biocontrol and the biocontrol agent may have to be in decline before it produces the secondary metabolites such as alkyl pyrones (Claydon *et al.*, 1987) that enable biocontrol. Certainly our results indicate that successful biocontrol action is associated only with a transient growth of *Trichoderma* during which there is no massive increase in biomass even though cfus may increase.

*Acknowledgements*—We are grateful to the USDA, ARS and the AFRC Underwood Funds for the award of fellowships to RDL, and to the AFRC for a studentship to JPC. We are also grateful to Margaret Ousley for excellent technical help and Cathy Townley for statistical advice.

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